Quantification of Horizontal Transmission of *Salmonella enterica* Serovar Enteritidis Bacteria in Pair-Housed Groups of Laying Hens

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An important source of human salmonellosis is the consumption of table eggs contaminated with *Salmonella enterica* serovar Enteritidis. Optimization of the various surveillance programs currently implemented to reduce human exposure requires knowledge of the dynamics of *S.* Enteritidis infection within flocks. The aim of this study was to provide parameter estimates for a transmission model of *S.* Enteritidis in laying-type chicken flocks. An experiment was carried out with 60 pairs of laying hens. Per pair, one hen was inoculated with *S.* Enteritidis and the other was contact exposed. After inoculation, cloacal swab samples from all hens were collected over 18 days and tested for the presence of *S.* Enteritidis. On the basis of this test, it was determined if and when each contact-exposed hen became colonized. A transmission model including a latency period of 1 day and a slowly declining infectivity level was fitted. The mean initial transmission rate was estimated to be 0.47 (95% confidence interval [CI], 0.30 to 0.72) per day. The reproduction number \( R_0 \), the average number of hens infected by one colonized hen in a susceptible population, was estimated to be 2.8 (95% CI, 1.9 to 4.2). The generation time, the average time between colonization of a “primary” hen and colonization of contact-exposed hens, was estimated to be 7.0 days (95% CI, 5.0 to 11.6 days). Simulations using these parameters showed that a flock of 20,000 hens would reach a maximum colonization level of 92% within 80 days after colonization of the first hen. These results can be used, for example, to evaluate the effectiveness of control and surveillance programs and to optimize these programs in a cost-benefit analysis.

Salmonellosis is one of the most common food-borne bacterial causes of human gastroenteritis worldwide (14, 19), and *Salmonella enterica* serovar Enteritidis is the serovar most frequently isolated from human cases of salmonellosis (13). Poultry products, especially table eggs, were identified as a predominant source of *S.* Enteritidis (e.g., references 8, 11, 20, and 22). Reduction of the number of *S.* Enteritidis-contaminated eggs or egg contamination in flocks of laying hens is a main target for reduction of human salmonellosis.

Various surveillance programs are currently carried out worldwide (e.g., references 5 and 23). These programs aim to identify colonized flocks and subsequently remove these flocks from the egg supply. It is unknown, however, how quickly after introduction of the bacteria into the flock and after colonization of the first laying hen the flock will be detected in these programs. In addition, it is not clear how many contaminated eggs may have been produced by this colonized flock before it is detected.

An evaluation of these programs is therefore necessary. A measure of the effectiveness of a surveillance program could be the number of contaminated eggs produced before detection, which depends on the interval between colonization of the first laying hen in the flock and detection. The amount of time until detection, in turn, depends not only on the reliability of the detection method used but also, above all, on the dynamics of the infection within the flock. Therefore, to optimize surveillance programs or to evaluate current programs for their effectiveness (e.g., references 10 and 16), parameters describing the population dynamics of the infection should be determined.

Quantifying transmission in commercial flocks is rather difficult, as it is unknown if and when during the production period *S.* Enteritidis is introduced, and it would require a high sampling frequency and large numbers of flocks to provide sufficient power for this determination. Therefore, we carried out transmission experiments with the aim of quantifying *S.* Enteritidis transmission between laying hens. We did this experimentally, with pairwise-housed laying hens, to capture three basic parameters of transmission: the mean level of infectiousness as measured by the reproduction ratio \( R_0 \), the individual variability in infectiousness related to excretion of *S.* Enteritidis in feces, and the mean time between subsequent generations of infection (generation time \( T_g \)).

The results can be used in future studies, for instance, in development and evaluation of surveillance programs. In ad-
dition, we will illustrate what our estimates mean for large flocks by simulating outbreaks in laying flocks.

MATERIALS AND METHODS

Animals. One hundred eighty laying hens, aged 17 weeks, of the Lohmann brown classic \(n = 90\) and Lohmann selected leghorn classic \(n = 90\) breeds were obtained from two commercial grower poultry farms in The Netherlands. The hens were not vaccinated against Salmonella spp. The farms were tested serologically for the presence of Salmonella antibodies on randomly selected hens and were found not to be suspected of previous Salmonella infections before the purchase of the experimental hens. After an acclimatization period of 4 days, cloacal swabs were taken from 90 hens in the entire trial on two consecutive days and subsequently bacteriologically tested for the presence of Salmonella spp. No Salmonella spp. were detected before the start of the trial.

Bacterial inoculum. The Salmonella strain used in this experiment was a nalidixic acid- and novobiocin-resistant S. Enteritidis phage type 4 (PT4) strain (as confirmed by the Dutch National Institute for Public Health and the Environment), courtesy of Paul Barrow (2, 3). The strain was taken from the –80°C freezer, transferred serially thrice in buffered peptone water (BPW) (Biokar Diagnostics, Pantin Cedex, France), and used for inoculation at a dose of 10⁹ CFU/ml.

Experimental design. Two replicates (called Exp 1 and Exp 2 hereafter) of one experiment were carried out. The latter had more-extensive sampling and a longer study period in order to allow for closer study of the infection chain. For each experiment, 90 hens were randomly allocated to three rooms with 30 birds each. Each room contained 40 cages. The cage sides and the cage floors were covered with plastic-coated carton. The cage floor was 0.5 m by 0.5 m and was covered with litter.

All hens were individually tagged. In each room, 10 pairs of hens were put in a cage. Each pair consisted of one white and one brown hen, producing white and brown eggs, respectively. In this way, it was possible to allocate each egg to the individual hen, which was needed for evaluation of immunological tests using egg yolk (M. E. Thomas, D. Klinkenberg, A. A. Bergwerff, E. van Eerden, J. A. Stegeman, and A. Boma, unpublished data). Also, in each room, 10 sentinel (5 brown and 5 white) were placed in the cages between the pairs to monitor cross-infection between cages and to demonstrate the independence of the experimental units (i.e., the cages). In order to avoid direct contact between pairs and sentinels, one cage was left empty in between each pair of animal-containing cages (Fig. 1).

On day −1 of the experiment, one randomly selected hen from each pair was removed and kept separate in a cage in the same room. These hens were inoculated in the esophagus with 1 ml of the S. Enteritidis PT4 inoculum. One day later, all inoculated hens were placed back into their original cages (day 0). So, each pair consisted of one inoculated and one contact-exposed bird (in-contact), either brown or white. In total, equal numbers of white and brown hens were inoculated or contact exposed. The sentinels were not inoculated.

Hens were provided with a commercial laying ration (Novex leg HP; de Heus BV, Ede, The Netherlands) without any antibiotics and tap water in troughs and were kept at 15 h, with 14 h of lighting. Feed and tap water were available ad libitum. Each batch of feed was pelleted at 76°C, treated with 2 kg/1,000 kg formaldehyde (Sal CURB Dry; Kemin Agrifoods, Herentals, Belgium), and tested for the presence of Salmonella in accordance with ISO protocol 6579:2002 (15). The experiment was conducted in a disease containment building at the experimental facilities of the Farm Animal Health Department, Faculty of Veterinary Medicine, Utrecht University, and approved by the Animal Welfare Committee of Utrecht University under contract number 2007.II.01.004.

Sampling. Cloacal swabs from both inoculated and in-contact hens and sentinels were taken on days 1 to 9, 11 to 14, and 16 and 18; in Exp 2, more samples were taken on other days, but these were not used for statistical analyses. Sentinels were sampled first, followed by sampling of the paired hens, in which the in-contact hen was always sampled first. Each cage was sampled using new arm gloves and aprons. Cloacal swabs were put into tubes containing 10 ml sterile BPW (Biokar Diagnostics) at room temperature and were transported for bacteriological examination.

Eggs were collected on days –1 and 26 and stored at 4°C. If a hen was not in production on day –1, collection started as soon as the first egg was produced. At the end of the experiment, on day 26 for Exp 1 and on days 32 (room 1), 39 (room 2), and 45 (room 3) in the case of Exp 2, all hens were euthanized by cervical dislocation.

Bacteriology. To detect Salmonella shedding of hens before inoculation, cloacal swabs were cultured by following the procedures recommended by ISO 6579:2002.

To detect Salmonella shedding after inoculation, cloacal swabs were enriched in BPW (18 ± 2 h, 37°C) and cultured on Rappaport-Vassiliadis medium (semi-solid modification) with 0.02 g/liter novobiocin (CM910; Oxoid, Basingstoke, United Kingdom) (24 ± 3 h, 42°C ± 1°C). Negative cultures were reincubated for a further 24 ± 3 h. Regular confirmation of positive colonies was performed by biochemical confirmation on tryptone soy agar and urea agar and lysine decarboxylase medium (Biotrading, Mijdrecht, The Netherlands) and serum agglutination (Pro-Lab Diagnostics, Ninton, United Kingdom).

S. Enteritidis PT4 and an in-house Escherichia coli K-12 strain were used as positive and negative controls, respectively, throughout the bacteriological procedures.

Immunology. Egg samples for antibody detection were prepared as follows. Each egg yolk was put in sterile petri dishes. From each egg yolk, 200 μl was collected and prediluted 1:5 (vol/vol) in 10 mM HEPES buffer at pH 7.4, containing 3 mM EDTA, 0.15 M sodium hydrochloride, 0.005% (vol/vol) surfactant P20 (Bisocore AB, Uppsala, Sweden), 0.85 M additional sodium chloride (Merck, Darmstadt, Germany), 1% (mass/vol) carboxymethylated dextran (Fluka Chemie, Buchs, Germany), and 0.05% (vol/vol) Tween 80 (Merck). These samples were stored at −20°C.

Samples were analyzed using an enzyme-linked immunosorbent assay for detection of chicken egg yolk antibodies against lipopolysaccharide of S. Enteritidis (Animal Health Service, Deventer, The Netherlands). In total, 79 (Exp 1) and 78 (Exp 2) combined day −1 and day 26 egg yolk samples were available.

S. Enteritidis transmission model. In a group of laying hens, S. Enteritidis-colonized birds excrete bacteria into the litter, after which these bacteria might be ingested by all birds in the group. Some of these ingestions are successful and result in colonization if the ingesting bird was not yet colonized. The rate (per day) at which bacteria from a single colonized bird \(i\) infected at \(\tau = 0\) are successfully ingested will be denoted by \(\beta(x)\). In other words, the colonization rate \(\beta(x)\) is the expected number of colonization events per day (resulting from bird \(i\) at time point \(\tau\) after colonization of bird \(j\)) in a large, susceptible population \(N\). From the perspective of the susceptible birds in a group of size \(n\), this means that each of them is colonized at a rate of \(\beta(x)n\), because the colonization events are shared among all chickens. Thus, in a pairwise experiment with a stocking density similar to that in a commercial flock, the susceptible chickens experience a colonization rate of \(\beta(x)n/2\). Because of this, it is possible to use the outcome of the pairwise experiments to provide an estimate for the parameters of \(\beta(x)\) and extrapolate the results to larger flocks (26).

We assume that, after a latency period of 1 day, the colonization rate will be constant from \(\tau = 1\) until \(\tau = \tau_i\), at level \(\beta_i\), after which it will gradually decrease at a rate of \(\gamma\) (Fig. 2a). This would reflect a short period for establishing the colonization \(\(\tau = 1\), a period with a high excretion level \(\(\tau = \tau_i\), and, finally, lower excretion levels and reduction of the infectiousness of environmental S. Enteritidis:
The mean of all \( R_i \) values will be denoted by \( R \), which is defined as the mean initial colonization rate day^{-1}. Similarly, the mean of all colonization rates \( [\beta(\tau)] \) will be denoted by \( \beta(\tau) \).

The total number of chickens that is expected to be colonized as a result of all bird excreted by chicken \( i \), the individual reproduction number \( (R_i) \), can be calculated from \( \beta(\tau) \) as

\[
R_i = \int_0^{\tau_i} \beta(\tau)d\tau = \beta_i \left( \tau_i - 1 + \frac{1}{\gamma} \right) \tag{2}
\]

Two important epidemiological parameters can be calculated from this model. The first is the basic reproduction number, defined as the average number of birds that will be colonized by a single colonized bird in a large, susceptible population \((1, 6)\). The basic reproduction number \((R_0)\) is the mean of all individual \( R_i \) values:

\[
R_0 = \bar{R}_i \tag{3}
\]

The second parameter is \( T_g \), the generation time, which is the average time between colonization of a "primary" hen and colonization of other hens by bacteria excreted by this "primary" hen \((9)\). \( T_g \) is the mean of the normalized colonization rate function (Fig. 2); in the final model (with \( \tau_i = 1 \)), the individual variation in \( R_i \) can be quantified, which is especially important in early development of Salmonella outbreaks in flocks \((6, 18)\).

**Estimation of S. Enteritidis transmission parameters.** In support of the decision to combine results from both experiments and breeds, we tested for (i) differences between the numbers of culture-positive samples in inoculated birds (Student’s \( t \) test) and (ii) differences in the proportions of colonized contact birds (Pearson’s \( \chi^2 \) test).

The transmission analysis aimed at estimating the parameters of \( \beta(\tau) \), with the initial colonization rate \( \beta_0 \) possibly related to two indicators of infectivity for the inoculated hen: the time of the first culture-positive sample \((T_i)\) and the total number of culture-positive samples \((X_i)\):

\[
\beta_i = b_0 + b_1X_i + b_2T_i \tag{4}
\]

Equations 1 and 4 were combined to construct the log likelihood function (see appendix S1 in the supplemental material).

\[
l(b_0, b_1, b_2, \gamma, \tau_1) = \sum_i k_i \log [\beta_i(u_i)/2] - \int_0^{\tau_1} \beta_i(\tau)/2d\tau \tag{5}
\]

with \( k_i = 1 \) if in-contact hen \( i \) was colonized (and \( k_i = 0 \) if not) and \( u_i \) the time between colonization of the inoculated bird and colonization of the in-contact bird, 1 day before the first culture-positive sample was obtained. Equation 5 was maximized to obtain maximum likelihood estimates. By including and excluding \( b_1 \) and \( b_2 \) from equation 4 and possibly setting \( \gamma \) to 0 or \( \tau_1 \) to 0 in equation 1, 12 models were fitted and the final model was selected by means of the corrected Akaike information criterion (AICc) \((4)\). The confidence intervals (CIs) for the parameters of the final model were derived by the profile likelihood method \((7)\).

From the final model, the mean initial colonization rate \( \beta(0) \) was estimated at \( b_0 + b_2X \) (where \( X \) is the mean \( X_i \) value), and the basic \( R_0 \) value was estimated as \( b/\gamma \). The CIs for \( \beta \) and \( R_0 \) were derived by the profile likelihood method, by use of the sum of the transmission log likelihood value (equation 5) and a log likelihood value for \( X \) (see appendix S2 in the supplemental material):

\[
h(X) = \sum_i \log[d_i(X_i)] \tag{6}
\]

in which \( d_i \) is the density of the normal distribution with a mean of \( X \). \( T_g \) was estimated as \( 1 + 1/\gamma \).

Student’s \( t \) test and Pearson’s \( \chi^2 \) test were carried out with Microsoft Excel 2003 (Microsoft Corporation). The transmission analysis was done with Mathematica 6.0 (Wolfram Research, Inc.).

**RESULTS**

**Descriptive analyses.** In total, 58 out of 60 inoculated hens tested culture positive at least once between day 1 and day 18. Of the remaining two, one was positive on day 0 (1 day postinoculation), and both were positive for antibodies on day 26, from which we concluded that inoculation had been successful in all 60 hens. The mean number of positive cultures was 6.8 (standard deviation, 3.6), and the numbers did not differ significantly between Exp 1 and Exp 2 \((P = 0.97)\) or between breeds \((P = 0.25)\). Forty-one of the 60 in-contact hens (23 and 18 in Exp 1 and 2, respectively) became culture positive at least once \( (\text{no difference } P = 0.16指导下)\). Out of these 41 positive contacts, 19 (11 and 8 in Exp 1 and 2, respectively) were the brown breed and 22 (12 and 10 in Exp 1 and 2, respectively) the white breed \((P = 0.41)\).

Of the inoculated hens, 37/48 (18/24 and 19/24 in Exp 1 and 2, respectively) developed antibodies in egg yolk against S. Enteritidis, detectable on day 26, as did 18/53 of the in-contact hens (9/27 and 9/26 in Exp 1 and 2, respectively). Three inoculated hens and one in-contact were found to be antibody positive before the experiment. Those hens were not in production at the time of inoculation, and their first egg used as a reference had been collected within a week after inoculation. The pairs with these four hens were not included in the transmission analysis.
Four sentinels out of 60 had culture-positive test results. One of these became culture positive on day 12, much later than for in-contact hens in neighboring cages, and showed a positive result for the egg yolk immune test on day 26. Another bird showed a positive result for the egg yolk immune test on day 1 only; no bacteria were found. Considering that a far larger proportion of in-contact hens had a positive culture than the sentinels and considering that 56 sentinels remained negative in each test, we concluded that the infection of in-contact hens was caused by the inoculated hens in the same cage and that the infection events in the cages are independent observations.

Estimation of *S. Enteritidis* transmission parameters. For the transmission analysis, the 56 pairs without immunologically positive samples on day 1 were used. Figure 3a shows the distribution of the number of positive cultures from inoculated hens, of which the mean ($X$) was 6.8. In the transmission analysis, parameters of the colonization rate function $\beta(\tau)$ were estimated, using equation 5. The best model (with the lowest AIC) was

$$\beta(\tau) = (b_0 + b_1X)\exp(-\gamma \tau)$$  (7)

which was without $T_\gamma$ and without $\tau_i$. In Fig. 2b, the mean colonization rate $\beta(\tau)$ is depicted: after the latency period of 1 day, without transmission, there is no period of constant colonization rate but, rather, an immediate exponential decrease. The change in infectiousness, each chicken expected to infect about one to five other chickens in a large population of susceptible birds. Finally, $T_g$ was estimated at 7.0 days (95% CI, 5.0 to 11.6 days).

To give an idea of what these estimates mean for the dynamics of *S. Enteritidis* in a flock of 20,000 laying hens, Fig. 4 shows the increase in the number of colonized laying hens after colonization of the first hen, one curve for the point estimates, and 100 curves for random parameter sets within the 95% confidence interval.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline colonization rate $b_0$</td>
<td>0.13 (0–0.40)</td>
</tr>
<tr>
<td>Additional colonization rate per</td>
<td>0.051 (0.014–0.094)</td>
</tr>
<tr>
<td>positive sample</td>
<td></td>
</tr>
<tr>
<td>Rate of decline of environmental</td>
<td>0.17 (0.094–0.25)</td>
</tr>
<tr>
<td>infectiousness</td>
<td></td>
</tr>
<tr>
<td>Mean no. of positive samples $X$</td>
<td>6.8 (5.8–7.7)</td>
</tr>
<tr>
<td>Mean initial colonization rate $\beta$</td>
<td>0.47 (0.30–0.72)</td>
</tr>
<tr>
<td>Basic reproduction ratio $R_0$</td>
<td>2.8 (1.9–4.2)</td>
</tr>
<tr>
<td>Generation time $T_g$</td>
<td>7.0 (5.0–11.6)</td>
</tr>
</tbody>
</table>

FIG. 3. (a) Distribution of $X$, the number of culture-positive samples of the inoculated hens; (b) distribution of $R_0$, based on the parameter estimates of the final model and the observed distribution of $X$.

FIG. 4. Courses of *S. Enteritidis* outbreaks in a flock of 20,000 laying hens. The black curve is simulated with the point estimates of the final model for the colonization rate. The 100 gray curves are simulated with random parameters within the 95% joint confidence area for the model parameters.
confident area. It is observed from the central curve that in a flock of 20,000 hens, 3% of the laying hens will be colonized 1 month after colonization of the first hen and 86% 1 month later and that a level of 92% will be reached another 20 days later. The remaining curves show the sensitivity of the S. Enteritidis dynamics to the uncertainty of the parameter estimates. It appears that the major increase in prevalence may occur 10 days earlier or 20 days later and that the final colonization level will be between 80% and 98%.

DISCUSSION

The aim of the study was to quantify the transmission of S. Enteritidis in laying hens. $R_0$ was estimated to be 2.8, and $T_g$ was estimated to be 7.0 days. These transmission characteristics imply that in a flock of 20,000 hens 92% of the laying hens would be colonized within 80 days after colonization of the first hen in a barn production flock.

The variation in infectiousness between hens, linked to the number of culture-positive samples, has also been quantified: individual reproduction ratios ranged from about 1 to 5 (Fig. 3b), which means that the most-infectious chickens are five times as infectious as the least. Although the exact range is difficult to interpret at this stage, the variability quantified in this paper will be of great value in evaluating surveillance programs. Variation in general will affect especially the initial stages of Salmonella outbreaks (18). More variation in $R_0$ increases the possibility that an introduction of Salmonella into a flock does not result in an outbreak, and it will affect the time between Salmonella introduction and outbreak detection by means of surveillance.

We estimated the initial transmission rate ($\beta$) for laying hens (after a 1-day latency period) at 0.47 day$^{-1}$ on average ($b_0 + b_x X$), which subsequently decreased slowly. Heres et al. (12) estimated the transmission rate ($\beta$) for broilers to be 1.15 day$^{-1}$. A difference was also observed in $R_0$ values: 2.8 (95% CI, 1.9 to 4.1) in laying hens (this study) and >4.8 in broilers. As these are large differences and the experimental setups were not very different, we think these differences are likely due to inherent differences between broilers and laying hens, like age or breed. This has been demonstrated, for example, for age- and breed-dependent susceptibility to colonization with E. coli O157:H7 for broilers and layers (21). Further studies are needed to corroborate these differences, as they could have important consequences for surveillance and control in the two poultry sectors. It would be interesting to obtain parameter estimates from field studies, as, for example, done for Campylobacter jejuni in broiler flocks (25).

We chose a pairwise design with two breeds and not a random mixing group homogeneous with respect to breed. By this design, we knew which chicken was infected by which, which provided more-detailed information on the infectivity of Salmonella. For instance, it allowed us to parameterize the infectivity function shown in Fig. 2a, with the flexibility of a constant high infectivity level for a long time followed by rapid decline or a slowly declining infectivity level; the latter appeared to fit better, as shown in Fig. 2b. It also allowed us to study the relation between the (variable) shedding of S. Enteritidis, expressed as the number of culture-positive samples of inoculated hens, and the infection rate. This may provide more insight in the interpretation of shedding patterns and the link with infectiousness, and it gives an estimate of the inherent variability between chickens in their reproduction ratio ($R_0$), as explained above.

Two more assumptions of the infectivity function (Fig. 2a) that was used to fit the data are worth discussing. First, a latency period of 1 day was assumed, because it is realistic to assume a period between inoculation and the start of infectiousness. The present experiment, where the inoculated chickens were placed with the susceptible chickens after 1 day, did not allow us estimate infectiousness before day 1. If there were no latency period and the infectiousness curve of Fig. 2b was extrapolated from $\tau = 1$ to $\tau = 0$, this would result in an $R_0$ value of 3.3 and a $T_g$ value of 6.0.

The second assumption involves the slowly decreasing infectiousness (Fig. 2a) instead of a fixed infectiousness level ($\beta$) during an infectious period with a beginning and an end (as, for example, in the work of Heres et al. [12] and many other experimental studies [17, 24, 26]). This is done to acknowledge the fact that Salmonella is transmitted via the environment. The environment can remain infectious for a long time after the infectious bird has stopped shedding bacteria, but the infectivity of the environment decreases. By modeling transmission via a contaminated environment, which is more realistic than doing so by direct contacts between chickens, we again hope to be better equipped when evaluating surveillance programs in future studies.

The results from our study will provide useful scientific background for S. Enteritidis control strategies for laying hens. The parameter estimates can be applied in development of surveillance studies for detection of S. Enteritidis, as, for example, done for bovine herpesvirus type 1 (10) and classical swine fever virus (16). Moreover, our study contributed more insight into the link between shedding patterns and infectiousness. The development of surveillance programs using our parameters may help to reduce the number of S. Enteritidis-contaminated eggs, which will result in reduction of human salmonellosis.

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